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Time-dependent aggrecan gene expression of articular chondrocytes in response to hyperosmotic loading

G. D. Palmer*, P.-h. G. Chao*, F. Raia*, R. L. Mauck*, W. B. Valhmu† and C. T. Hung*
**Cellular Engineering Laboratory, Department of Biomedical Engineering, Columbia University, New York, NY 10027, U.S.A.; †Biosciences Laboratory, Division of Orthopaedic Surgery, University of Wisconsin Medical School, Madison, WI 53792, U.S.A.*

Summary

Objective: To investigate the effects of increasing extracellular osmolality on aggrecan gene expression and cell size in cultured chondrocytes.

Design: Aggrecan promoter activity and mRNA levels were measured in bovine monolayer chondrocytes subjected to hyperosmotic loading for different time periods, using transient transfection assays or RT-PCR. Cell size changes were also determined using an epifluorescence microscopy system.

Results: Hyperosmotic loading for 24 h suppressed aggrecan promoter activity and mRNA levels approximately two-fold. However no suppression of promoter activity was observed when exon 1 was deleted from the human aggrecan promoter construct. Osmotic regulation of aggrecan gene expression was time-dependent and found to correlate with cell shrinking and swelling. No suppression in promoter activity was observed when the hyperosmotic stimulus was applied in a cyclic manner, or when serum was present in the culture medium.

Conclusion: Hyperosmotic loading regulates aggrecan gene expression and cell size in isolated chondrocytes. Osmotic regulation of gene expression is also affected by the time-varying nature of loading and the presence of serum. © 2001 OsteoArthritis Research Society International

Key words: Aggrecan, Osmolality, Cartilage, Chondrocyte.

Introduction

The unique load-bearing properties of articular cartilage depend on the structural composition and ultrastructural organization of the specialized connective tissue, particularly the interaction between collagens and proteoglycans of the extracellular matrix (ECM)[1,2]. Aggrecan is the large aggregating proteoglycan of articular cartilage and each aggrecan molecule contains over a hundred highly sulfated, negatively charged glycosaminoglycan (GAG) chains covalently attached to its protein core. The functional properties of aggrecan reside in its ability to concentrate negative charges, giving rise to the tissue's highly negative fixed charge density (FCD), as well as form macromolecular aggregates with hyaluronan[3,4]. Articular cartilage thus contains high cation concentrations and tissue osmolality relative to other body fluids, ~380/450 mOsm/kgH_2O[5]. Joint loading of articular cartilage leads to matrix deformation and fluid flow within the soft hydrated tissue. The combination of fluid flow and deformation-induced changes in FCD expose chondrocytes to osmotic changes in their milieu. These osmotic variations represent one important physical stimulus in the regulation of chondrocyte activities[6].

The synthesis, incorporation and degradation of ECM proteins is orchestrated by chondrocytes which are in turn regulated by environmental factors. It has been well established that mechanical forces can regulate the metabolic activity of chondrocytes in articular cartilage (see Guilak *et al.*[7] for a comprehensive review). *In vivo* studies using animal models for joint disuse or surgically-induced joint instability have shown that altered mechanical loading results in cartilage pathology. Changes in aggrecan synthesis[8–11] and increased aggrecan degradation[8–11] have been identified as important events in these models. Re-establishing the mechanical environment by joint remobilization in the disuse model reverses the suppression of aggrecan synthesis and restores the mechanical properties of cartilage[12]. A number of *in vitro* studies have been performed to investigate how mechanical signals control aggrecan expression and synthesis. Deformational loading studies using cartilage explants demonstrate that long-term static compression of cartilage suppresses aggrecan synthesis[13,14] while short-term cyclic compression at specific frequencies can stimulate aggrecan synthesis[14–16]. Mechanical loading of articular cartilage also generates a complex series of physical events at the tissue and cellular level which could potentially modulate chondrocyte biosynthetic activity[6]. Studies have shown that hydrostatic

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Address correspondence to: Clark T. Hung, Ph.D., Cellular Engineering Laboratory, Department of Biomedical Engineering, Columbia University, 407 CEPSR MC8904, 530 West 120th Street, New York, NY 10027, U.S.A. Tel: 212-854-6542; Fax: 212-854-8725; E-mail: cth6@columbia.edu

pressure[17,18], fluid flow[19,20] and pH[21] can modulate aggrecan synthesis but their interaction in determining the overall cellular response remains unclear.

Static loading of articular cartilage also causes fluid expression and increased proteoglycan concentration surrounding the chondrocyte which leads to alterations in ionic and osmotic composition of the tissue. Changes in extracellular osmolality have also been shown to affect chondrocyte biosynthetic activities[5,22–25]. Urban *et al.*[25] reported maximal proteoglycan and protein synthetic rates at physiologic osmolarity (350–450 mOsm) in cartilage and isolated chondrocytes. Increasing or decreasing the media osmolality outside this range (by different amounts of sucrose or NaCl addition) led to a fall in proteoglycan synthesis rates. Chondrocyte swelling and shrinkage have also been reported in cartilage and isolated cells in response to hypo- and hypertonic shock[22,26,27]. Since cell shape is known to influence chondrocyte phenotypic expression[28], cell deformation due to osmotic swelling and shrinkage may be a potential mechanism through which proteoglycan synthesis is regulated in response to osmotic loading. Alterations in intracellular ionic composition have also been suggested to be responsible for the fall in proteoglycan synthesis rates[25]. This is supported by the observed stimulation of ion transporters such as the Na^+-K^+-$2Cl^-$[29] cotransporter and the Na^+/H^+ exchanger[30] in response to increased osmolality and cell shrinkage.

Despite these findings, the molecular mechanisms involved in osmotic regulation of aggrecan synthesis are not known. Previous *in vitro* studies have demonstrated regulation of aggrecan mRNA levels in response to a number of physical stimuli including static compression[31–33] and hydrostatic pressure[17,18]. We have previously demonstrated that fluid-flow induced shear-stress of bovine articular chondrocytes down-regulates aggrecan gene expression at the level of promoter activity using a reporter construct containing a 2.4 kb promoter fragment and exon 1 of the human aggrecan gene[19]. Analysis of this portion of the gene has revealed several putative mechanoresponsive elements (SSREs)[33] which may mediate transcriptional regulation in response to fluid-flow and other physical stimuli. In this study we hypothesize that this region of the aggrecan gene is similarly involved in the regulation of aggrecan gene expression in response to osmotic loading of isolated chondrocytes. We also investigated the correlation between aggrecan gene expression and chondrocyte cell size in response to osmotic changes. A better understanding of the molecular mechanisms involved in osmotic regulation of aggrecan gene expression may help elucidate the mechanisms that underlie chondrocyte mechanotransduction.

Methods

PREPARATION OF HUMAN AGGRECAN PROMOTER CONSTRUCTS

A 1.7-kb fragment, corresponding to the distal portion of the human aggrecan promoter, was cloned into the *Mlu*I/*Nde*I site of a luciferase reporter construct pAGC1(−701)/5UTR[19], which contained the proximal 701-bp promoter region and exon 1 (5′-UTR). This generated the construct pAGC1(−2368)/5UTR. A second promoter construct containing a deletion in exon 1 was made by digesting pAGC1(−2368)/5UTR with *PpuM*1 to generate pAGC1(−2368/+25) (Fig. 1).

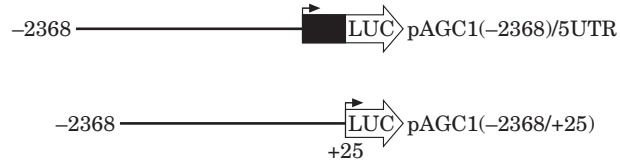

Fig. 1. Schematic of human aggrecan promoter constructs used in transfection studies. A construct containing a 2368-bp promoter fragment and exon 1 of the human aggrecan gene was made by cloning a 1.7-kb upstream promoter fragment into the previously made pAGC1(−701)/5UTR[33]. This pAGC1(−2368)/5UTR construct was used as a template to generate an exon 1 deletion construct, pAGC1(−2368/+25), which had 350 nucleotides removed from the end of exon 1.

CELL CULTURE

Bovine articular cartilage was harvested from the carpometacarpal joint of 4- to 6-month-old calves within 6 h of sacrifice and enzymatically digested with hyaluronidase and collagenase, as previously described[19], to isolate chondrocytes. Chondrocytes were plated at a density of 5×10^5 cells per 35 mm dish. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, amino acids (0.5× minimum essential amino acids, 1× non-essential amino acids), buffering agents (10 mM Hepes, 10 mM TES, 10 mM BES) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) in a 5% CO_2 incubator at 37°C until experiments. The osmolality of this medium was measured using a freezing point depression osmometer (Advanced Instruments Inc, Norwood, MA) and determined to be 360 mOsm. Similar to reports by other investigators[28], cultured chondrocytes in our study exhibited positive type II collagen staining and cuboidal morphology, in addition to exhibiting aggrecan gene expression, throughout the duration of our investigations.

TRANSIENT TRANSFECTION

Primary chondrocytes were transiently transfected with 3 μg of aggrecan promoter construct and 0.75 μg of β-gal cotransfection plasmid per 35 mm dish, 24 h after plating, using the ProFection calcium phosphate mammalian transfection kit (Promega Corporation, Madison, WI). After transfection for 48 h, cells were lysed and luciferase and β-galactosidase activities were determined as previously described[19].

QUANTITATION OF mRNA LEVELS

For RNA quantitation, total RNA was extracted from untransfected chondrocytes, 72 h after plating, using the RNAgents Total RNA isolation system (Promega), which is based on the method of Chomczynski and Sacchi[34]. The isolated RNA was further purified using the RNeasy Kit (Qiagen Inc., Valencia, CA), and the levels of aggrecan mRNA were quantified as previously described[33,35].

OSMOTIC LOADING

Isolated chondrocytes were incubated with solutions of different osmolality for various time points in a 37°C, 5% CO_2 incubator. The solutions were prepared by adding increasing amounts of sucrose, NaCl or polyethylene glycol

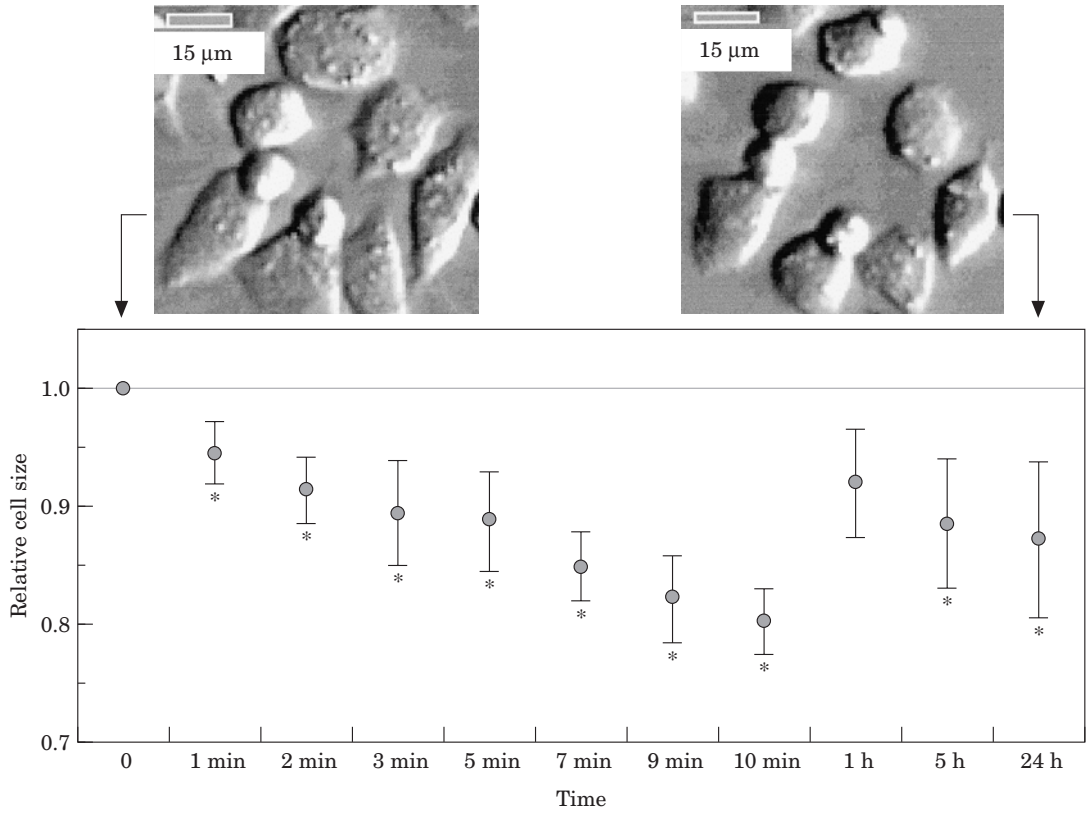

Fig. 2. The effect of hyperosmotic loading on chondrocyte cell size. Images of cells were recorded and cell borders were manually outlined for 10 cells (1–10 min) or 55 cells (1–24 h) at each time point using MetaMorph software. Representative Hoffman modulation contrast images for t=0 and t=24 h are shown. Cell areas for each time point were normalized to their initial size at t=0. The values for hyperosmotically loaded cells (580 mOsm) are expressed relative to isotonic controls (360 mOsm) for each time point. *Indicates significant difference relative to initial cell size at t=0.

(PEG) to fully supplemented DMEM without serum. The osmolality of this medium was also 360 mOsm. The osmolality was then adjusted from standard DMEM to give a range of 360 mOsm to 680 mOsm by solute addition. Studies were performed with sucrose as the solute unless otherwise stated. After osmotic loading either aggrecan promoter activity, mRNA levels or cell size measurements were determined.

CELL SIZE MEASUREMENTS

In order to determine the effect of osmolality on cell size, digital images of chondrocytes subjected to osmotic shock were recorded using an epifluorescence microscopy system. The system consisted of an Olympus 1X−70 inverted microscope, and a MicroMax 5-Mhz interline transfer chip camera (Princeton Instruments, Trenton, NJ). Hoffman modulation contrast images were recorded using a 10× objective (0.683 pixel/μm) and MetaMorph software (Universal Imaging Corporation, NJ). Monolayer cells were then placed under the microscope 48 h after plating and subjected to osmotic shock. For static (continuous) osmotic loading digital images were recorded at regular time intervals up to 24 h. For dynamic loading, images were acquired every 5 min. Using 400× digital magnification, cell borders were manually outlined for at least 10 cells (loaded and control) using MetaMorph software. The precision of this measurement was determined to be ~1.4%. The cell area of each cell over time (t) was normalized by its original cell area (t=0). The cell area ratios for hyperosmotically loaded cells (over time) were then normalized to the mean cell area ratio (over time) of isotonic controls.

STATISTICAL ANALYSIS

Comparisons were performed on all data using either paired or unpaired t-test, one sample t-test, or one way analysis of variance (ANOVA) with the Student–Newman–Keuls multiple range comparison test with α=0.05. For cell size comparisons, statistical analysis was performed on raw data for 0–10 min groups whereas normalized data was compared for t>10 min groups.

Results

EFFECT OF OSMOTIC LOADING ON CHONDROCYTE CELL SIZE

To investigate the effect of medium osmolality change on cell size, the cell size of cultured chondrocytes exposed to hyperosmotic (580 mOsm) media was measured over a period of 24 h (Fig. 2). Exposure to hyperosmotic media decreased average chondrocyte cell size by 5–20% over the first 10 min (P<0.0001 to P=0.011, N=10–55), but within 1 h cell size recovered significantly (P<0.0001 relative to 10 min, N=55) to its original size. However, no further recovery was observed after 1 h and average cell size remained decreased by 12–13% compared to initial

size (P<0.0001). The same cell size changes were observed with hyperosmotic media made by NaCl addition instead of sucrose (data not shown). Although direct measurements of osmotic loading-induced cell volume changes were not performed, a positive correlation between change in cell area and change in peak cell height (Pearson correlation coefficient r=0.53, P=0.03, N=16) was determined using confocal microscopy of representative cells. Relative changes to peak cell height were greater than changes to cell area.

OSMOTIC LOADING REGULATES AGGRECAN GENE EXPRESSION

Previous studies have demonstrated that osmolality regulates proteoglycan synthesis rates in isolated chondrocytes[25]. To determine if osmolality regulates aggrecan gene expression directly, aggrecan promoter activity and mRNA levels were measured in cultured chondrocytes after incubation with hyper-osmotic media for 24 h.

To investigate aggrecan promoter activity, chondrocytes were transfected with a chimeric luciferase reporter construct, pAGC1(−2368)5UTR, containing 2.4 kb of the human aggrecan promoter and exon 1 (5′-UTR). Hyperosmotic loading with 580 and 680 mOsm media significantly decreased activity of pAGC1(−2368)/5UTR by 60–70% (P<0.0001) [Fig. 3(A)]. The effect of osmotic loading on aggrecan promoter activity was also tested using a second reporter construct, pAGC1(−2368/+25), which contained a truncated exon 1 (5′-UTR). This portion of the aggrecan gene has previously been shown to contain important regulatory regions that modulate basal and regulated promoter expression[19,33,36]. In contrast to pAGC1(−2368)/5UTR, hyperosmotic loading with either 580 or 680 mOsm media did not significantly change activity of pAGC1(−2368/+25) relative to control cultures [Fig. 3(A)].

The effect of hyperosmotic loading on pAGC1(−2368)/5UTR activity was also investigated using growth media containing NaCl and PEG [Fig. 3(B)]. Adjusting the media osmolality to 580 mOsm by PEG or NaCl addition had similar effects to sucrose, causing a significant 50–60% suppression of pAGC1(−2368)/5UTR activity.

EFFECT OF OSMOLALITY ON AGGRECAN mRNA LEVELS

To determine if osmolality also regulates aggrecan steady-state mRNA levels, quantitative PCR was performed on RNA extracted from chondrocytes cultured in hyperosmotic (580 mOsm) media for 24 h. Hyperosmotic loading suppressed aggrecan mRNA levels compared with 360 mOsm controls [Fig. 3(C)], but the magnitude of suppression was approximately 15% lower than promoter activity. The observed suppression of aggrecan mRNA levels is consistent with our aggrecan promoter studies.

EFFECT OF RECOVERY FROM HYPER-OSMOTIC LOADING

To investigate if there is a correlation between osmotic regulation of chondrocyte cell size and aggrecan gene expression we determined cell size and aggrecan promoter activity over a 24 h period of loading and then for a further 24 h recovery period. Recovery was performed by incubating cultured cells with control 360 mOsm media (DMEM) following the 24 h period of hyperosmotic loading with 580 mOsm media. Chondrocyte cell size and aggrecan

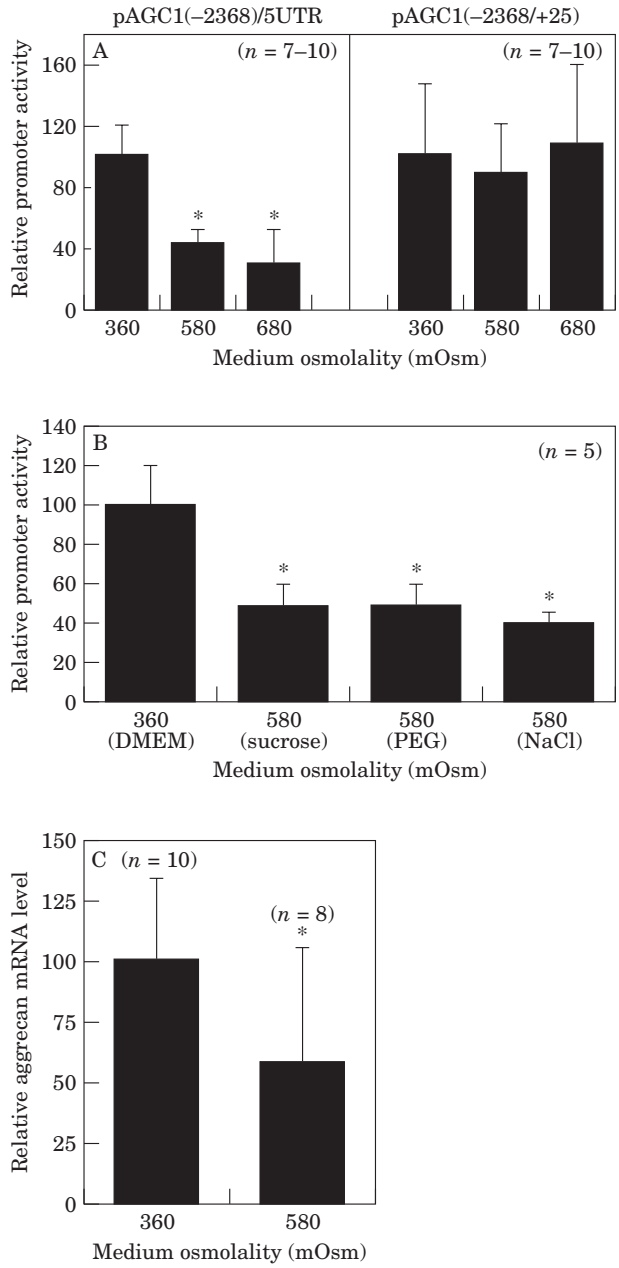

Fig. 3. (A) The effect of hyperosmotic loading on activity of the human aggrecan promoter. Primary monolayer chondrocytes were transiently transfected with an aggrecan promoter-luciferase constructs, pAGC1(−2368)/5UTR or pAGC1(−2368/+25). After transfection, cells were incubated with DMEM or hyperosmotic DMEM prepared by addition of increasing amounts of sucrose. In all promoter activity studies luciferase activity was normalized to β-galactosidase activity for each sample. Values in each group were expressed relative to 360 mOsm controls. (B) The effect of hyperosmotic loading on aggrecan promoter activity using PEG and NaCl as osmolytes. Activity of pAGC1(−2368)/5UTR was determined in response to 24 h of loading with various hyperosmotic media (580 mOsm). (C) Effect of hyperosmotic loading on aggrecan mRNA levels. Total RNA was extracted from chondrocytes after 24 h of osmotic loading with 580 mOsm media (sucrose). The mRNA copy number was calculated from each sample and normalized to total RNA[35]. For each experiment, data from each treatment group were expressed as a % of 360 mOsm.

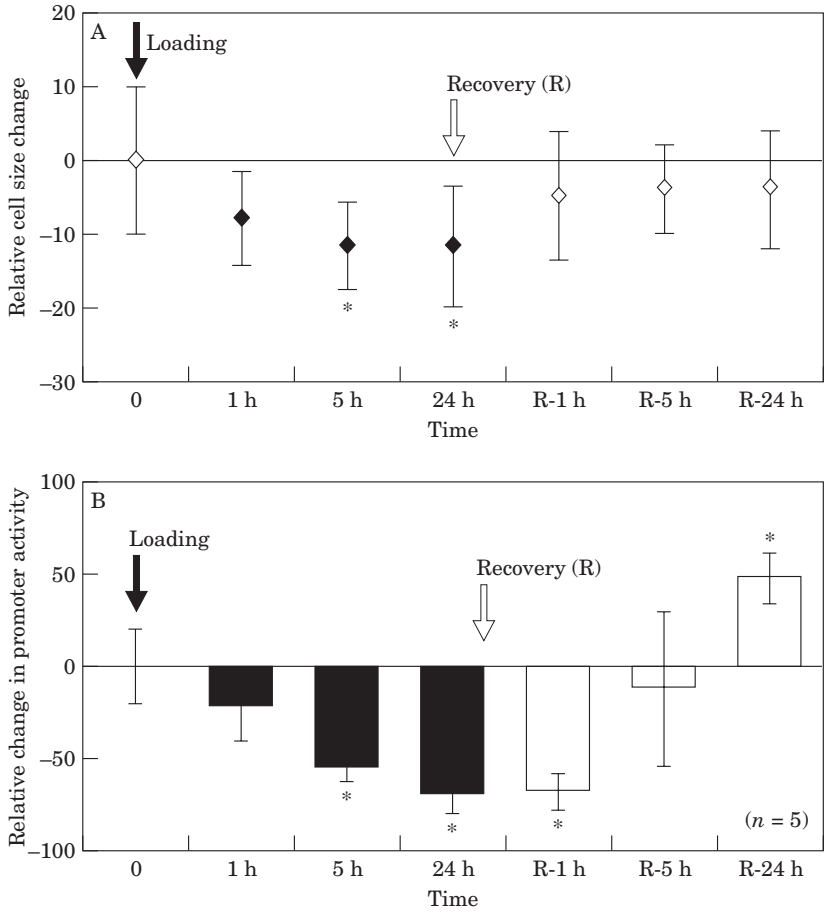

Fig. 4. Chondrocyte cell size (A) and aggrecan promoter activity (B) following removal of hyperosmotic loading. Chondrocytes were incubated with 580 mOsm media (A: —◆—; B: ■) at t=0 and after 24 h osmolality was decreased by changing media to 360 mOsm (A: —◇—; B: □). Control cultures were incubated with 360 mOsm only at 0 and 24 h. Values for cell size and promoter activity were expressed as % change from controls. *indicates significant difference relative to initial cell size at t=0.

promoter activity were determined during hyperosmotic loading and after the osmolality change (Fig. 4). As previously, chondrocyte cell size was not significantly decreased until after 1 h of hyperosmotic loading (P<0.0001, N=55) [Fig. 4(A)] with a 50% suppression, relative to unloaded controls, in aggrecan promoter activity observed after 5 h [Fig. 4(B), P<0.0001]. However, within 1 h of incubation with 360 mOsm media following hyperosmotic loading chondrocytes recovered to within 5% of their original size, P>0.05, N=25–55 [Fig. 4(A)]. Incubation with 360 mOsm media also affected aggrecan promoter activity. Within 5 h, promoter activity was no longer suppressed compared with unloaded controls and by 24 h activity was 48% higher than controls [Fig. 4(B), P<0.0001].

We further investigated the effects of cell swelling and shrinkage by subjecting monolayer chondrocytes to dynamic osmotic loading by alternating media osmolality between 360 and 580 mOsm for repeated cycles (Fig. 5). The osmotic loading protocols consisted of complete solution changes every 10 minutes (360–580–360, etc.), i.e. at a frequency of 0.0017 Hz. In control cultures repeated additions of 360 mOsm were performed to account for potential shear effects. Figure 5(A) depicts the normalized cell size for 10 cells analysed in the same field of view. Chondrocytes were found to shrink and re-swell in response to intermittent loading at 580 and 360 mOsm, while no cell size changes were observed in 360 mOsm controls. The cell size immediately before and after each change in medium [indicated by arrows in Fig. 5(A)] was significantly different (P<0.0002–0.013, N=10). Therefore chondrocytes can regulate their size in response to repeated cycles of hyperosmotic loading. In transfected cultures, dynamic loading also stimulated aggrecan promoter activity 20% over controls, whereas static or continuous loading for the same period decreased promoter activity 20% [Fig. 5(B), P<0.05].

EFFECT OF SERUM ON THE OSMOTIC REGULATION OF CHONDROCYTE CELL SIZE AND AGGRECAN GENE EXPRESSION

In order to further characterize the response of chondrocytes to osmotic loading we investigated the effect of the presence of serum (10% FBS) in the media on chondrocyte cell size and aggrecan gene expression. In contrast to serum-free conditions, chondrocyte cell size decreased more gradually when serum was present [Fig. 6(A)], and no recovery was observed after 1 h. After 24 h, cell size had significantly decreased the same extent as serum-free cultures (approximately 14%), but the cell morphology was more flattened and fibroblast-like compared to a spherical/cuboidal appearance in DMEM (not shown). Serum also

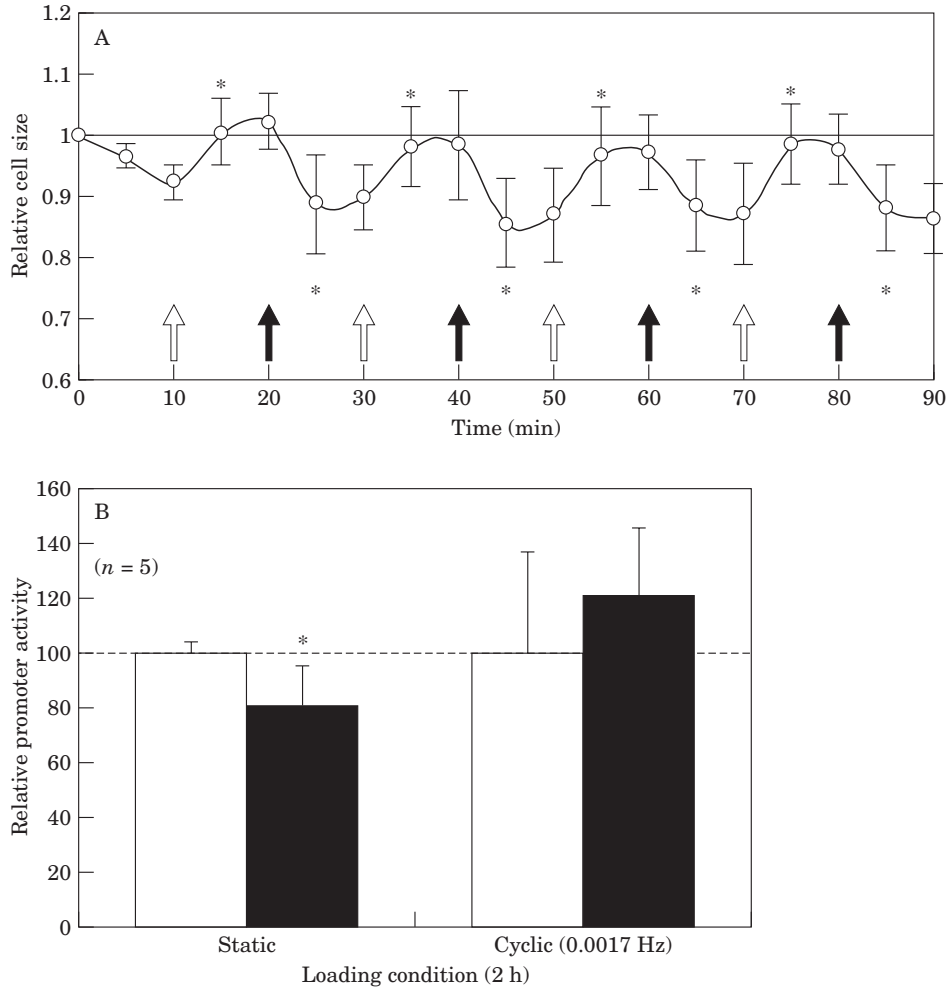

Fig. 5. Effect of dynamic osmotic loading on chondrocyte cell size (A) and aggrecan promoter activity (B). The dynamic loading protocol consisted of complete solution changes every 10 min alternating between 360 and 580 mOsm for a 2 h period. In unloaded cultures 360 mOsm media were added every 10 min to account for possible shear-induced effects on the cells during the loading period. Static or continuous loading at 580 mOsm was also performed on transfected cells in parallel with dynamically loaded cultures. Cell size measurements and transfections were performed as before. Values for cell size and promoter activity were normalized to 360 mOsm controls. *indicates significant difference relative to previous time point. Unloaded (360 mOsm), □; loaded (580 mOsm), ■.

affected osmotic regulation of aggrecan gene expression. Loading with 580 mOsm media containing 10% FBS did not reduce activity of pAGC1(−2368)/5UTR [Fig. 6(B)]. Similarly, FBS blocked the down-regulation of aggrecan mRNA levels. In related studies to assess the contribution of serum proteins in this response, experiments were performed with DMEM supplemented with bovine serum albumin, comprising the major protein component of serum. Sucrose addition to DMEM plus 0.1% BSA (580 mOsm) resulted in a decreasing trend of aggrecan promoter activity that was significant at 24 h. The normalized promoter activity at 1 h was 1.09±0.43 (N=5), for 5 h 0.908±0.27 and for 24 h 0.59±0.35 (P<0.0001). The cells exhibited size changes similar in trend to that for DMEM.

Discussion

Mechanical loading of cartilage imposes a number of changes on the physical environment of a chondrocyte, including alterations in ionic and osmotic composition. Although the osmotic environment within cartilage during loading has not been directly measured, it has been suggested that changes in extracellular osmolality and ion concentration are partly responsible for the fall in proteoglycan synthesis rates in response to static compression[23]. To learn more about chondrocyte response to osmotic loading as well as mechanotransduction, we subjected isolated chondrocytes to levels and durations of differential osmotic loading that have previously been shown to affect chondrocyte morphology and biosynthetic activity[5,22,24–27,37–39] and investigated the effects on cell size and aggrecan gene expression.

In chondrocytes, the passive water flux across the membrane occurs very rapidly[38,40] and osmotic regulation occurs slowly, indicating that chondrocytes behave as perfect osmometers[25,41]. In the present study, chondrocyte cell size was decreased in response to hyperosmotic loading, but cells were significantly larger at 24 h than after 10 min, indicating that some volume recovery took place (Fig. 2). Similar studies have shown that isolated chondrocytes do not effectively regulate their volumes after short-term (1 h) hyperosmotic loading[5,26], although recovery to

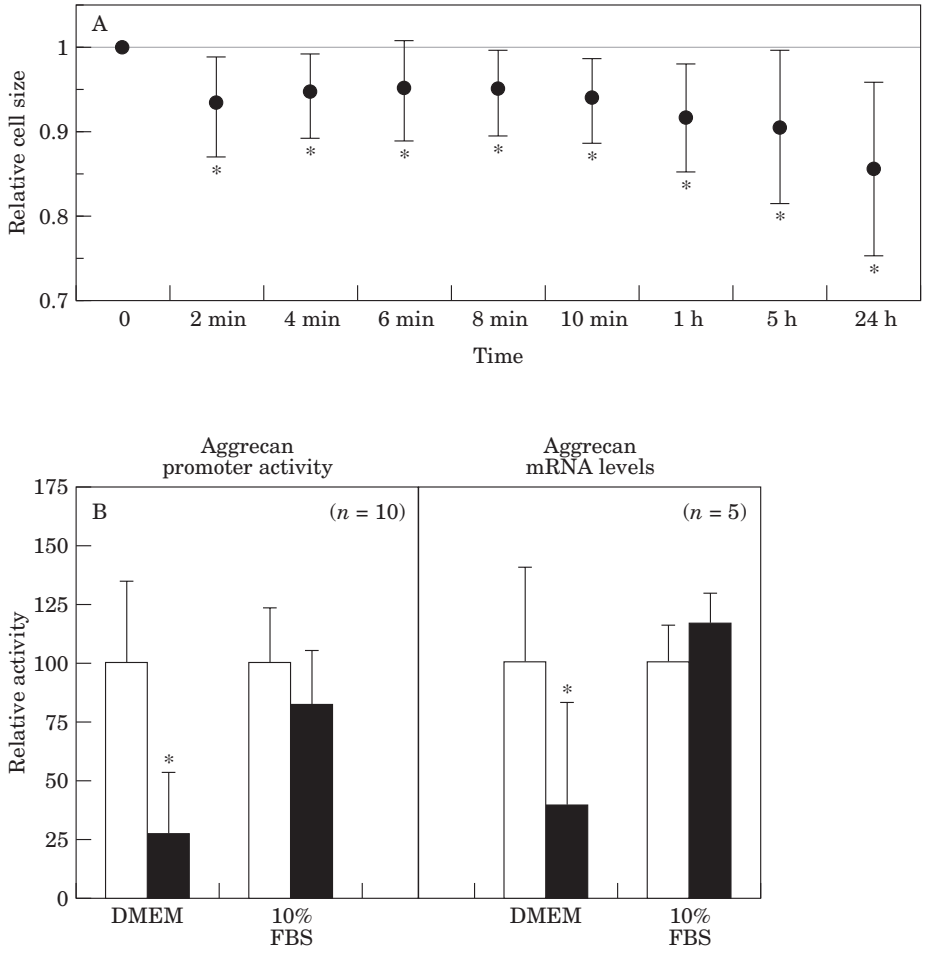

Fig. 6. The effect of serum on osmotic regulation of chondrocyte cell size (A) aggrecan gene expression (B). Static osmotic loading was performed on monolayer chondrocytes for 24 h either in the presence or absence of 10% FBS. Cell size measurements, aggrecan mRNA levels and promoter activity were determined as with serum-free conditions. Cell size decreased significantly at all time points ($P<0.0001$ to $P=0.026$) relative to initial cell size ($t=0$). In contrast to serum-free cultures, no recovery was observed at 1 h after hyperosmotic loading. B: 360 mOsm, □; 580 mOsm, ■.

initial size has been reported after 24 h in isolated chondrocytes[5], and after 80 min in cartilage explants[27]. The specific event(s) that give rise to the observed transcriptional changes to aggrecan gene expression observed after 24 h of hyperosmotic loading are unclear. Potential stimuli that may contribute to this response include the initial passive flux of water out of the cell (e.g., 0–10 min in Fig. 2), the early successful regulatory volume increase (RVI) efforts by the cells (e.g., 10 min to 1 h in Fig. 2), the later unsuccessful efforts by the cells for RVI (e.g., 1–5 h in Fig. 2), and the final new cell shrunken equilibrium (e.g., 5–24 h, Fig. 2). The role of these different stimuli will be elucidated in the future through the examination of different loading durations, frequency and osmotic differential magnitude.

Our initial efforts to vary the frequency of osmotic loading by application of a cyclic osmotic loading applied at 0.0017 Hz revealed a minor stimulation of aggrecan promoter activity, whereas static loading for the same period suppressed promoter activity (Fig. 5). This result indicates that exposure to a hyperosmotic environment may be stimulatory to aggrecan gene expression when the osmotic stimulus is applied intermittently. This osmotic stimulus may be more physiologically relevant as chondrocytes are likely to be subjected to cyclical changes in their osmotic environment *in vivo*. Several authors have noted differing responses of chondrocytes following either cell shrinkage or swelling with respect to volume regulation[26,36], K^+ channel activation[29], and calcium mobilization[37,39]. It is likely that successive cell swelling and shrinking induced by dynamic loading will affect these cellular processes. However, the duration, magnitude and rate of the applied osmotic stimulus are likely to be major factors, as they are for other physical stimuli, in determining the overall effect on chondrocyte biosynthetic activity.

Our studies also demonstrated that hyperosmotic loading regulates aggrecan gene expression, as determined by cell transfection studies with the aggrecan promoter construct, pAGC1(−2368)/5UTR, and quantitation of aggrecan mRNA levels (Fig. 3). These findings are consistent with the suppression of proteoglycan synthesis rates observed in isolated chondrocytes when media osmolality was raised above 300 mOsm[25]. Furthermore, activity of a truncated promoter construct containing an exon 1 deletion, leaving a 5′ flanking region of −2368 to +25, was not suppressed by hyperosmotic loading [Fig. 3(A)]. Our findings suggest that transcriptional regulation through specific gene sequences may be a major mechanism through which chondrocyte

genes are regulated in response to osmotic changes. Prior sequence analysis of the deleted region of exon 1 has revealed the presence of potential osmotic responsive elements[53] (G. Palmer, unpublished data). These include three putative SSREs, which are known to modulate transcriptional activity in response to mechanical stimuli[42], and an STRE which modulates gene regulation in *Saccharomyces cerevisiae* in response to osmotic shock[43]. Further deletion analysis of exon 1 will identify the osmotic responsive elements that modulate aggrecan gene expression in response to hyperosmotic loading.

To further characterize the chondrocyte response to osmotic loading we investigated the effect of restoring the extracellular osmolality to physiological levels (DMEM) after hyperosmotic loading (Fig. 4). Cell size recovered to within 5% of initial size almost immediately and this was accompanied by a slower rebound of aggrecan promoter activity which was eventually elevated 50% above unloaded controls after 24 h. This experiment could also be viewed as a single cycle of a 1.2×10^{-5} Hz dynamic loading stimulus. A 'rebound' of proteoglycan synthesis rates to control levels has been observed within an 8–48 h period following removal of static compressive loading in cartilage disks[13,14,44]. The stimulation after 24 h observed in our studies using isolated chondrocytes may be explained by the lack of regulatory influences of the ECM. ECM depletion in cultured chondrocytes has led to a stimulation of proteoglycan synthesis[45] and aggrecan mRNA expression[46]. Nevertheless the observed correlation between cell size and aggrecan promoter activity suggests that osmotic changes in cell morphology directly regulate expression of the aggrecan gene. Several studies have demonstrated a relationship between phenotypic expression and chondrocyte cell shape in cytoskeletal components rather than actual cell-shape[47–49]. Osmotic shock of chondrocytes in the range of 480–580 mOsm has been shown to alter chromatin and cytoskeletal structure, as well as morphology of the endoplasmic reticulum[22]. These intracellular changes could potentially affect the transcriptional and translational processes involved with aggrecan gene expression.

There is evidence to suggest that the responses of cells to mechanical stimuli are modulated by biological agonists such as serum[50–52]. Since serum is often used in tissue culture for *in vitro* cartilage and chondrocyte studies, we investigated the effects of serum on the chondrocyte response to osmotic loading. The presence of fetal bovine serum blocked osmotic regulation of aggrecan gene expression but did not affect cell shrinkage (Fig. 6). The effect on gene expression is likely to be due to the action of biochemical mediators (e.g. growth factors) in serum, since replacement with albumin (0.1% BSA)—a major serum protein—did not block osmotic regulation of aggrecan promoter activity. As serum is also known to stimulate aggrecan gene expression in cell culture[53,54] (and unpublished data) it is not clear whether its effect on aggrecan gene expression in the present study is because of a masking effect due to saturating levels of serum, or direct inhibition of osmotic signaling mechanisms. We noted that a larger proportion of cells appeared more fibroblast-like when serum was present. Interestingly, a transition to a more fibroblast-like phenotype has been observed in chondrocytes cultured in serum-containing hyperosmotic media over a 2-week period[22] although it has not been determined if this leads to changes in biosynthetic activity or gene expression. The presence (and concentration) of serum may therefore be an important factor in determining the chondrocyte response to osmotic loading and perhaps other physicochemical stimuli. Our bovine serum albumin result implies that chemical mediators (e.g. growth factors) in serum are responsible for giving rise to the observed disparate response with respect to serum-free cultures.

The present study on isolated cells has extended the current understanding of how chondrocytes respond to hyperosmotic loading. We report that hyperosmotic loading-induced regulation of aggrecan gene expression in chondrocytes is modulated through a specific region of the gene. However, our findings suggest that the overall effect on gene expression is determined by factors such as the temporal nature of the loading and the presence of potential biological agonists, i.e., serum. Our preliminary studies[55] of chondrocytes encapsulated in agarose[28] subjected to 24 h of hyperosmotic loading have demonstrated aggrecan promoter activity results that parallel those reported here, and provide evidence that our observations are not due solely to the use of monolayer cultures. Although this aggrecan response appears to be similar, it remains to be determined if the same mechanisms (e.g. cell size change, cytoskeletal reorganization) govern the response under both culture conditions.

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